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CLONING AND PRODUCTION OF HUMAN ACETYLCHOLINESTERASE
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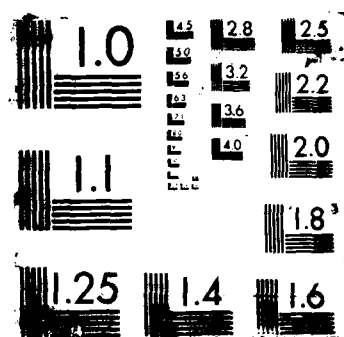
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ANNUAL SUMMARY REPORT

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December 1984

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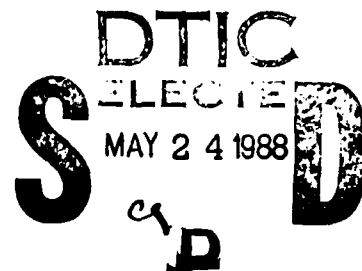
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The goal of this work was to clone by DNA recombinant technology the gene for acetylcholine esterase from a human source. In order to do this we prepared and purified monoclonal antibody from large amounts of medium in which HG-72 hybridoma (ATCC) has been grown. The monoclonal antibody was found to inhibit human acetylcholine esterase but not eel acetylcholine esterase. Attempts to determine an appropriate oligonucleotide sequence probe are also discussed. <i>Key words: Acetylcholinesterase. (HWS)</i>					

SUMMARY

The goal of this work was to clone by DNA recombinant technology the gene for acetylcholine esterase from a human source. In order to do this we prepared and purified monoclonal antibody from large amounts of medium in which HG-72 hybridoma (ATCC) has been grown. The monoclonal antibody was found to inhibit human acetylcholine esterase but not eel acetylcholine esterase. Attempts to determine an appropriate oligonucleotide sequence probe are also discussed.

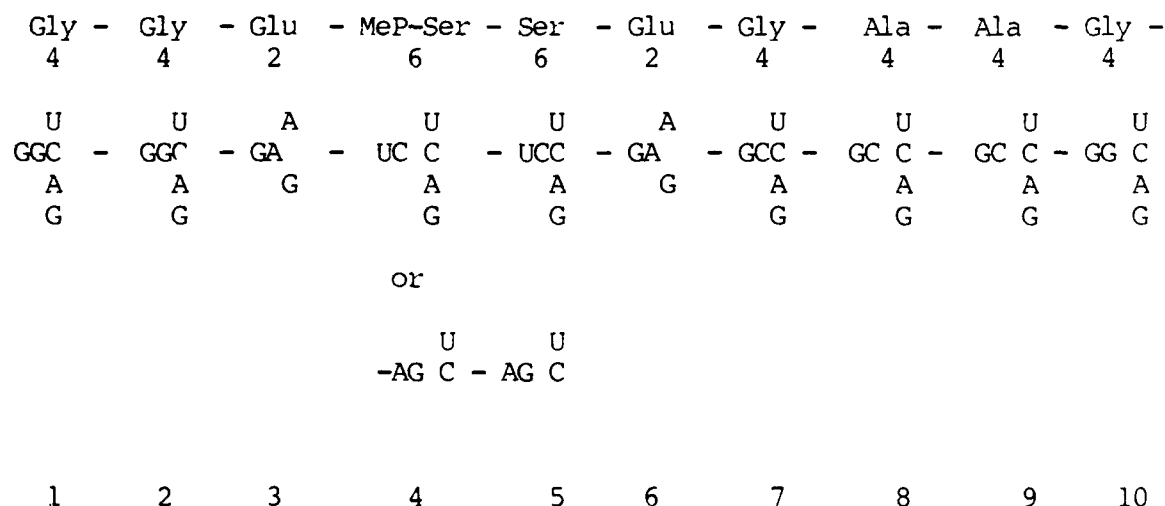
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1. Attempts to develop an oligonucleotide sequence probe.

One of the successful methods to isolate a message RNA is to use an oligonucleotide probe. Below is written the amino acid sequence in the region of the reactive residue of electric eel acetylcholinesterase as per Schaffer et al., (1973) Biochemistry 12, 2946 and the respective mRNA sequence. Under each amino acid is the number of triplet codons possible. As one can see the number of possible sequences would be astronomical for this decapeptide! It is not an enviable situation to be dealing with a region that is so highly degenerate.

Figure 1. AChE active site sequences



Regions of homology between the active site sequences of the enzyme and binding site sequences of the receptor were sought also. Since the paired serine residues are characteristic of the active site, three regions in the receptor with paired serine residues were located. Their flanking sequences revealed no apparent homology with those of the enzyme reactive site. Further, the paired serine sequences of the receptor do not allow us to choose

which nucleic acid sequences might be preferable for an oligo-nucleotide probe since we have UCC-AGC codons used for nucleotides 802-807, AGU-UCA used for nucleotide residues 859-864, and UCA-AGC for serine pair encoded at nucleotides 1,174-1,179 of the sequence. Lack of similarity is not surprising if we keep in mind that in the extreme, similarity in the receptor binding pocket with the enzyme would impart catalytic properties.

Since the Noda or Numa group felt that the cysteine groups in the 128-142 (amino acid) region were involved in binding, some paper chemistry was performed (Fig. 2).

Figure 2 - Homology based on polarity

NP = non-polar
P = Polar
NC = Neg charge
PC = Positive charge

Receptor amino acid sequence (putative binding)

<u>P</u>	<u>NC</u>	<u>NP</u>	<u>NP</u>	<u>NP</u>	<u>P</u>	<u>PC</u>	<u>NP</u>	<u>NP</u>	<u>NP</u>
128	129	130	131	132	133	134	135	136	137
Cys	Glu	Ile	Ile	Val	Thr	His	Phe	Pro	Phe

<u>NC</u>	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>	<u>P</u>
138	139	140	141	142	143
Asp	Glu	Glu	Asn	Cys	Thr

Esterase amino acid sequence (putative catalytic)

<u>P</u>	<u>P</u>	<u>NC</u>	<u>P</u>	<u>P</u>	<u>NC</u>	<u>P</u>	<u>NP</u>	<u>NP</u>	<u>NP</u>
1	2	3	4	5	6	7	8	9	10
Gly	Gly	Glu	Ser	Ser	Glu	Gly	Ala	Ala	Gly

We substituted the amino acid sequence of the enzyme with their respective class assignments based on polarity of their R-groups and ran that sequence alongside those in the 128-142 region of receptor looking for homology based

on polarity. This revealed a common non-polar region three peptides long:
(135-136-137) receptor and

Phe-Pro-Phe
(Ala-ala-Gly) AChE. By itself this is not very encouraging, but it may be wiser to use a stretch of five amino acids including these three rather than using a stretch which drops us in the quagmire of the highly degenerate serine region. In that case, we could use the pentapeptide Glu-Gly-Ala-Ala-Gly and by omitting the last nucleotide in the corresponding sequence, a trick used by the Kyoto group, our possibilities would narrow down to 128. The Kyoto group had the luxury of choosing a region where three of the five codons had a degeneracy of two.

As for now, the numbers did not permit us to make a more definite choice for a sequence probe. Since this approach of seeking an oligonucleotide probe did not seem very promising, we decided to continue pursuing the monoclonal antibody to HACE described in our first Annual Report.

2. Purification and Characterization of Monoclonal Antibody from HG-72

Hybridoma.

Hybridoma HB-72 (ATCC) was used as the source of antihuman acetylcholinesterase antibody. Growth of these cells and purification of the antibody is described in our July 1983 Annual Summary Report. Our purification usually started from 1 liter of serum free medium in which the HB-72 cells were placed 25-30 hrs before harvesting the medium. To obtain such a volume of medium required growing a large amount of cells which was no small or easy task.

To identify the antibody from hybridoma HG-72 as an anti-acetylcholinesterase, the following was done:

(1) Double Immunodiffusion: A 1% agar gel in 5 mM potassium phosphate

buffer, pH 7.2, containing 0.15 M NaCl was made on clean glass slides. Uniform wells were cut using a gel puncher and samples were applied into individual wells. The slides were placed in a moist chamber at 4°C overnight. Then the agar plates were pressed under several layers of filter paper and a piece of heavy wooden board (10 g/cm²) for 15 min. The gels were washed extensively in 0.15 M NaCl solution for 6-8 hr at 4°C and stained with coomassie brilliant blue.

The result: No precipitation was detected. This result was probably due to the Antibody-Antigen complex not being large enough to precipitate in the gel.

- (2) Immunoprecipitation: Protein A conjugated sepharose 4B (SPA Pharmacia) was used as affinity gel. Protein A-Sepharose CL4B is protein A covalently coupled to CL-4B by the cyanogen bromide method. 7.5 mg SPA which contains 53 µg protein A was swollen in 0.1 M potassium phosphate buffer, pH 8.0 for two hours and centrifuged at 150 x g for 10 min at room temperature. The supernatant was discarded and 1 mg crude antibody was added to the gel. The mixture was gently shaken at 4°C overnight. SPA-antibody (SPA-Ab) complex was collected by centrifugation at 4°C and washed three times with phosphate buffer. 50 µg human acetylcholinesterase was added to SPA-Ab complex and gently shaken at 4°C overnight. The mixture was centrifuged at 4°C and the supernatant was saved for enzymatic assay. The colorimatic assay of acetylcholinesterase was used according to the method described by George L. Ellman (Biochemical Pharmacology (1961) 7:88-95).

The SPA antibody gave us 95% inhibition of activity of HACE as shown by only 15% of the activity being measurable in the centrifuged supernatant after precipitation with the SPA-Ab complex.

3. Specificity

In order to check its specificity an Ouchterlony immunodiffusion was performed using antibody prepared from the HB72 hybridoma (5 mg protein/mls), eel acetylcholine esterase (1 mg/ml); 3% agarose and 20 mM NaCl/20 mM PO₄ buffer pH 7.8.

Antibody was added to center well of five hole Ouchterlony. Antigen was added to four of the outer holes. Diameter of well = 3 mm, distance between wells (measured from circumferences) = 5 mm. Agar slides were placed in 150 mm plastic petri dishes on top of buffer saturated whatman #1 filter paper. Dish was leveled and kept @ 4°C in cold room. After 24 hours, wells were topped off with respective Ab or Ag sample. Diffusion was allowed to run for 48 hours. Results:

HB /2 Ab X Eel AChE →→ no cross reaction

4. Comment

It took us much longer than expected to work up a useable preparation of this monoclonal antibody and prove its antibody specificity. By this time our contract had expired.

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